To gain insight into possible *in vivo* functions of FLRG, we analyzed FLRG and follistatin expression in various human tissues. FLRG was expressed at highest levels in the testis, adrenal glands, lung, heart and liver. Lower levels of FLRG transcripts were seen in the stomach, small intestine, colon, pancreas, thyroid, ovary and prostate. FLRG mRNA could not be detected in the thymus, kidney, skeletal muscle, liver, brain, peripheral blood lymphocytes and spleen. The expression pattern of follistatin mRNA revealed obvious similarities to that of FLRG, although this molecule was also expressed in the liver. The FLRG expression data extend the results obtained by Hayette et al. (Hayette, S., *et al., Oncogene* 16, 2949-54 (1998)), who found expression of this molecule in murine heart, lung, testis, and kidney. In contrast to these data, however, we could not detect FLRG mRNA in the human kidney.

To determine potential differences in the regulation of follistatin and FLRG expression, we analyzed the effect of various growth factors and cytokines on the expression of these genes in HaCaT keratinocytes. This cell line was chosen since it expresses fairly high levels of both follistatin and FLRG mRNA. Cells were rendered quiescent by serum starvation and treated with the epithelial mitogens KGF and EGF. Follistatin and FLRG mRNAs were hardly detectable in quiescent keratinocytes. Upon addition of the growth factors, a strong induction of follistatin expression was observed, whereby maximal levels were seen 8 hours after addition of the mitogen. EGF was the more potent inducer compared to KGF. This induction was long-lasting and follistatin mRNA levels had still not returned to basal levels 24 hours after growth factor stimulation. In contrast to follistatin, FLRG induction occurred already within one hour after addition of the growth factors. However, the degree of induction was significantly lower.

To determine whether follistatin and FLRG mRNA induction is specific for epithelial cell mitogens, we analyzed the effect of TGF-beta1, a strong inhibitor of keratinocyte proliferation. Interestingly, TGF-beta1 caused a strong induction of both FS and FLRG expression, whereby the degree of induction was similar for both genes. Similar as with EGF and KGF, induction of FLRG expression by TGF-beta1 occurred earlier compared to follistatin.

Taken together, these results demonstrate differential regulation of follistatin and FLRG mRNA expression, indicating that the two proteins might be available under

different biological circumstances. The discovery of a novel activin-binding protein with a different expression pattern compared to follistatin necessitates the reinterpretation of various activin expression data, since the presence of FLRG is likely to modulate the activity of the available activin. Whether this activin-FLRG interaction leads to inhibition of activin function as shown for follistatin remains to be elucidated.

Example 6: Production of an Antibody.

a) Hybridoma Technology

The antibodies of the present invention can be prepared by a variety of methods. (See, Current Protocols, Chapter 2.) As one example of such methods, cells expressing follistatin-3 is administered to an animal to induce the production of sera containing polyclonal antibodies. In a preferred method, a preparation of follistatin-3 protein is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

In the most preferred method, the antibodies of the present invention are monoclonal antibodies (or protein binding fragments thereof). Such monoclonal antibodies can be prepared using hybridoma technology. (Kohler et al., *Nature* 256:495 (1975); Kohler et al., Eur. J. Immunol. 6:511 (1976); Kohler et al., Eur. J. Immunol. 6:292 (1976); Hammerling, *et al.*, in: *Monoclonal Antibodies and T-Cell Hybridomas*, Elsevier, N.Y., pp. 563-681 (1981).) In general, such procedures involve immunizing an animal (preferably a mouse) with follistatin-3 polypeptide or, more preferably, with a secreted follistatin-3 polypeptide-expressing cell. Such cells may be cultured in any suitable tissue culture medium; however, it is preferable to culture cells in Earle's modified Eagle's medium supplemented with 10% fetal bovine serum (inactivated at about 56°C), and supplemented with about 10 g/l of nonessential amino acids, about 1,000 U/ml of penicillin, and about 100 ug/ml of streptomycin.

The splenocytes of such mice are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention; however, it is preferable to employ the parent myeloma cell line (SP2O), available from the ATCC. After fusion, the resulting hybridoma cells are selectively

maintained in HAT medium, and then cloned by limiting dilution as described by Wands et al. (Gastroenterology 80:225-232 (1981).) The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding the follistatin-3 polypeptide.

Alternatively, additional antibodies capable of binding to follistatin-3 polypeptide can be produced in a two-step procedure using anti-idiotypic antibodies. Such a method makes use of the fact that antibodies are themselves antigens, and therefore, it is possible to obtain an antibody which binds to a second antibody. In accordance with this method, protein specific antibodies are used to immunize an animal, preferably a mouse. The splenocytes of such an animal are then used to produce hybridoma cells, and the hybridoma cells are screened to identify clones which produce an antibody whose ability to bind to the follistatin-3 protein-specific antibody can be blocked by follistatin-3. Such antibodies comprise anti-idiotypic antibodies to the follistatin-3 protein-specific antibody and can be used to immunize an animal to induce formation of further follistatin-3 protein-specific antibodies.

It will be appreciated that Fab and F(ab')2 and other fragments of the antibodies of the present invention may be used according to the methods disclosed herein. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')2 fragments). Alternatively, secreted follistatin-3 protein-binding fragments can be produced through the application of recombinant DNA technology or through synthetic chemistry.

For *in vivo* use of antibodies in humans, it may be preferable to use "humanized" chimeric monoclonal antibodies. Such antibodies can be produced using genetic constructs derived from hybridoma cells producing the monoclonal antibodies described above. Methods for producing chimeric antibodies are known in the art. (See, for review, Morrison, Science 229:1202 (1985), Oi et al., BioTechniques 4:214 (1986); Cabilly et al., U.S. Patent No. 4,816,567; Taniguchi et al., EP 171496; Morrison et al., EP 173494; Neuberger et al., WO 8601533; Robinson et al., WO 8702671; Boulianne et al., Nature 312:643 (1984); Neuberger et al., Nature 314:268 (1985).)

b) Isolation of antibody fragments directed against follistatin-3 from a library of scFvs.

Naturally occuring V-genes isolated from human PBLs are constructed into a large library of antibody fragments which contain reactivities against follistatin-3 to which the donor may or may not have been exposed (see e.g., U.S. Patent 5,885,793 incorporated herein in its entirety by reference).

Rescue of the Library. A library of scFvs is constructed from the RNA of human PBLs as described in WO92/01047. To rescue phage displaying antibody fragments, approximately 10^9 *E. coli* harbouring the phagemid are used to inoculate 50 ml of 2xTY containing 1% glucose and 100 ug/ml of ampicillin (2xTY-AMP-GLU) and grown to an O.D. of 0.8 with shaking. Five ml of this culture is used to innoculate 50 ml of 2xTY-AMP-GLU, 2 x 10^8 TU of delta gene 3 helper (M13 delta gene III, see WO92/01047) are added and the culture incubated at 37°C for 45 minutes without shaking and then at 37°C for 45 minutes with shaking. The culture is centrifuged at 4000 r.p.m. for 10 min. and the pellet resuspended in 2 liters of of 2xTY containing 100 ug/ml ampicillin and 50 ug/ml kanamycin and grown overnight. Phage are prepared as described in WO92/01047.

M13 delta gene III is prepared as follows: M13 delta gene III helper phage does not encode gene III protein, hence the phage(mid) displaying antibody fragments have a greater avidity of binding to antigen. Infectious M13 delta gene III particles are made by growing the helper phage in cells harbouring a pUC19 derivative supplying the wild type gene III protein during phage morphogenesis. The culture is incubated for 1 hour at 37°C without shaking and then for a further hour at 37°C with shaking. Cells are spun down (IEC-Centra 8, 4000 revs/min for 10 min), resuspended in 300 ml 2xTY broth containing 100 ug ampicillin/ml and 25 ug kanamycin/ml (2xTY-AMP-KAN) and grown overnight, shaking at 37°C. Phage particles are purified and concentrated from the culture medium by two PEG-precipitations (Sambrook et al., 1990), resuspended in 2 ml PBS and passed through a 0.45 um filter (Minisart NML; Sartorius) to give a final concentration of approximately 10^{13} transducing units/ml (ampicillin-resistant clones).

Panning of the Library. Immunotubes (Nunc) are coated overnight in PBS with 4 ml of either 100 ug/ml or 10 ug/ml of a polypeptide of the present invention. Tubes are

blocked with 2% Marvel-PBS for 2 hours at 37°C and then washed 3 times in PBS. Approximately 10^{13} TU of phage is applied to the tube and incubated for 30 minutes at room temperature tumbling on an over and under turntable and then left to stand for another 1.5 hours. Tubes are washed 10 times with PBS 0.1% Tween-20 and 10 times with PBS. Phage are eluted by adding 1 ml of 100 mM triethylamine and rotating 15 minutes on an under and over turntable after which the solution is immediately neutralized with 0.5 ml of 1.0M Tris-HCl, pH 7.4. Phage are then used to infect 10 ml of mid-log *E. coli* TG1 by incubating eluted phage with bacteria for 30 minutes at 37 degree C. The E. coli are then plated on TYE plates containing 1% glucose and 100 ug/ml ampicillin. The resulting bacterial library is then rescued with delta gene 3 helper phage as described above to prepare phage for a subsequent round of selection. This process is then repeated for a total of 4 rounds of affinity purification with tube-washing increased to 20 times with PBS, 0.1% Tween-20 and 20 times with PBS for rounds 3 and 4.

Characterization of Binders. Eluted phage from the 3rd and 4th rounds of selection are used to infect *E. coli* HB 2151 and soluble scFv is produced (Marks, *et al.*, 1991) from single colonies for assay. ELISAs are performed with microtitre plates coated with either 10 pg/ml of the polypeptide of the present invention in 50 mM bicarbonate pH 9.6. Clones positive in ELISA are further characterized by PCR fingerprinting (see e.g., WO92/01047) and then by sequencing.

Example 7: Method of Determining Alterations in the Follistatin-3 Gene.

RNA isolated from entire families or individual patients presenting with a phenotype of interest (such as a disease) is be isolated. cDNA is then generated from these RNA samples using protocols known in the art (See, Sambrook.) The cDNA is then used as a template for PCR, employing primers surrounding regions of interest in SEQ ID NO:1. Suggested PCR conditions consist of 35 cycles at 95 degree C for 30 seconds, 60-120 seconds at 52-58 degree C, and 60-120 seconds at 70 degree C, using buffer solutions described in Sidransky, D., et al., Science 252:706 (1991)

PCR products are then sequenced using primers labeled at their 5' end with T4 polynucleotide kinase, employing SequiTherm Polymerase. (Epicentre Technologies).

The intron-exon borders of selected exons of follistatin-3 is also determined and genomic PCR products analyzed to confirm the results. PCR products harboring suspected mutations in follistatin-3 is then cloned and sequenced to validate the results of the direct sequencing.

PCR products of follistatin-3 are cloned into T-tailed vectors as described in Holton, T.A. and Graham, M.W., Nucleic Acids Research, 19:1156 (1991) and sequenced with T7 polymerase (United States Biochemical). Affected individuals are identified by mutations in follistatin-3 not present in unaffected individuals.

Genomic rearrangements are also observed as a method of determining alterations in the follistatin-3 gene. Genomic clones isolated according to methods well-known in the art are nick-translated with digoxigenindeoxy-uridine 5'-triphosphate (Boehringer Manheim), and FISH performed as described in Johnson, Cg. et al., Methods Cell Biol. 35.73-99 (1991). Hybridization with the labeled probe is carried out using a vast excess of human cot-1 DNA for specific hybridization to the follistatin-3 genomic locus.

Chromosomes are counterstained with 4,6-diamino-2-phenylidole and propidium iodide, producing a combination of C- and R-bands. Aligned images for precise mapping are obtained using a triple-band filter set (Chroma Technology, Brattleboro, VT) in combination with a cooled charge-coupled device camera (Photometrics, Tucson, AZ) and variable excitation wavelength filters. (Johnson, Cv et al., Genet. Anal. Tech. Appl., 8.75 (1991).) Image collection, analysis and chromosomal fractional length measurements are performed using the ISee Graphical Program System. (Inovision Corporation, Durham, NC.) Chromosome alterations of the genomic region of follistatin-3 (hybridized by the probe) are identified as insertions, deletions, and translocations. These follistatin-3 alterations are used as a diagnostic marker for an associated disease.

Example 8: Method of Detecting Abnormal Levels of Follistatin-3 in a Biological Sample

Follistatin-3 polypeptides can be detected in a biological sample, and if an increased or decreased level of follistatin-3 is detected, this polypeptide is a marker for a

particular phenotype. Methods of detection are numerous, and thus, it is understood that one skilled in the art can modify the following assay to fit their particular needs.

For example, antibody-sandwich ELISAs are used to detect follistatin-3 in a sample, preferably a biological sample. Wells of a microtiter plate are coated with specific antibodies to follistatin-3, at a final concentration of 0.2 to 10 ug/ml. The antibodies are either monoclonal or polyclonal and are produced by the method described in Example 6. The wells are blocked so that non-specific binding of follistatin-3 to the well is reduced.

The coated wells are then incubated for > 2 hours at RT with a sample containing follistatin-3. Preferably, serial dilutions of the sample should be used to validate results. The plates are then washed three times with deionized or distilled water to remove unbounded follistatin-3.

Next, 50 ul of specific antibody-alkaline phosphatase conjugate, at a concentration of 25-400 ng, is added and incubated for 2 hours at room temperature. The plates are again washed three times with deionized or distilled water to remove unbounded conjugate.

Add 75 ul of 4-methylumbelliferyl phosphate (MUP) or p-nitrophenyl phosphate (NPP) substrate solution to each well and incubate 1 hour at room temperature. Measure the reaction by a microtiter plate reader. Prepare a standard curve, using serial dilutions of a control sample, and plot follistatin-3 polypeptide concentration on the X-axis (log scale) and fluorescence or absorbance of the Y-axis (linear scale). Interpolate the concentration of the follistatin-3 in the sample using the standard curve.

Example 9: Formulation.

The invention also provides methods of treatment and/or prevention of diseases, disorders, and/or conditions (such as, for example, any one or more of the diseases, disorders, and/or conditions disclosed herein) by administration to a subject of an effective amount of a Therapeutic. By therapeutic is meant a polynucleotides or polypeptides of the invention (including fragments and variants), agonists or antagonists thereof, and/or antibodies thereto, in combination with a pharmaceutically acceptable carrier type (e.g., a sterile carrier).

The Therapeutic will be formulated and dosed in a fashion consistent with good medical practice, taking into account the clinical condition of the individual patient (especially the side effects of treatment with the Therapeutic alone), the site of delivery, the method of administration, the scheduling of administration, and other factors known to practitioners. The "effective amount" for purposes herein is thus determined by such considerations.

As a general proposition, the total pharmaceutically effective amount of the Therapeutic administered parenterally per dose will be in the range of about 1ug/kg/day to 10 mg/kg/day of patient body weight, although, as noted above, this will be subject to therapeutic discretion. More preferably, this dose is at least 0.01 mg/kg/day, and most preferably for humans between about 0.01 and 1 mg/kg/day for the hormone. If given continuously, the Therapeutic is typically administered at a dose rate of about 1 ug/kg/hour to about 50 ug/kg/hour, either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed. The length of treatment needed to observe changes and the interval following treatment for responses to occur appears to vary depending on the desired effect.

Therapeutics can be are administered orally, rectally, parenterally, intracistemally, intravaginally, intraperitoneally, topically (as by powders, ointments, gels, drops or transdermal patch), bucally, or as an oral or nasal spray. "Pharmaceutically acceptable carrier" refers to a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

Therapeutics of the invention are also suitably administered by sustained-release systems. Suitable examples of sustained-release Therapeutics are administered orally, rectally, parenterally, intracistemally, intravaginally, intraperitoneally, topically (as by powders, ointments, gels, drops or transdermal patch), bucally, or as an oral or nasal spray. "Pharmaceutically acceptable carrier" refers to a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include

intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

Therapeutics of the invention are also suitably administered by sustained-release systems. Suitable examples of sustained-release Therapeutics include suitable polymeric materials (such as, for example, semi-permeable polymer matrices in the form of shaped articles, e.g., films, or mirocapsules), suitable hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, and sparingly soluble derivatives (such as, for example, a sparingly soluble salt).

Sustained-release matrices include polylactides (U.S. Pat. No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma-ethyl-L-glutamate (Sidman et al., Biopolymers 22:547-556 (1983)), poly (2- hydroxyethyl methacrylate) (Langer et al., J. Biomed. Mater. Res. 15:167-277 (1981), and Langer, Chem. Tech. 12:98-105 (1982)), ethylene vinyl acetate (Langer et al., Id.) or poly-D- (-)-3-hydroxybutyric acid (EP 133,988).

Sustained-release Therapeutics also include liposomally entrapped Therapeutics of the invention (*see* generally, Langer, *Science* 249:1527-1533 (1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 317 -327 and 353-365 (1989)). Liposomes containing the Therapeutic are prepared by methods known per se: DE 3,218,121; Epstein et al., Proc. Natl. Acad. Sci. (USA) 82:3688-3692 (1985); Hwang et al., Proc. Natl. Acad. Sci.(USA) 77:4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese Pat. Appl. 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily, the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. percent cholesterol, the selected proportion being adjusted for the optimal Therapeutic.

In yet an additional embodiment, the Therapeutics of the invention are delivered by way of a pump (*see* Langer, *supra*; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)).

Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990)).

For parenteral administration, in one embodiment, the Therapeutic is formulated generally by mixing it at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. For example, the formulation preferably does not include oxidizing agents and other compounds that are known to be deleterious to the Therapeutic.

Generally, the formulations are prepared by contacting the Therapeutic uniformly and intimately with liquid carriers or finely divided solid carriers or both. Then, if necessary, the product is shaped into the desired formulation. Preferably the carrier is a parenteral carrier, more preferably a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, and dextrose solution. Non-aqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes.

The carrier suitably contains minor amounts of additives such as substances that enhance isotonicity and chemical stability. Such materials are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate, acetic acid, and other organic acids or their salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) polypeptides, e.g., polyarginine or tripeptides, proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone, amino acids, such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, mannose, or dextrins, chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counterions such as sodium; and/or nonionic surfactants such as polysorbates, poloxamers, or PEG.

The Therapeutic is typically formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml, preferably 1-10 mg/ml, at a pH of about 3 to 8. It will be understood that the use of certain of the foregoing excipients, carriers, or stabilizers will result in the formation of polypeptide salts.

Any pharmaceutical used for therapeutic administration can be sterile. Sterility is readily accomplished by filtration through sterile filtration membranes (e.g., 0.2 micron

membranes). Therapeutics generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Therapeutics ordinarily will be stored in unit or multi-dose containers, for example, sealed ampoules or vials, as an aqueous solution or as a lyophilized formulation for reconstitution. As an example of a lyophilized formulation, 10-ml vials are filled with 5 ml of sterile-filtered 1% (w/v) aqueous Therapeutic solution, and the resulting mixture is lyophilized. The infusion solution is prepared by reconstituting the lyophilized Therapeutic using bacteriostatic Water-for-Injection.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the Therapeutics of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the Therapeutics may be employed in conjunction with other therapeutic compounds.

The Therapeutics of the invention may be administered alone or in combination with adjuvants. Adjuvants that may be administered with the Therapeutics of the invention include, but are not limited to, alum, alum plus deoxycholate (ImmunoAg), MTP-PE (Biocine Corp.), QS21 (Genentech, Inc.), BCG, and MPL. In a specific embodiment, Therapeutics of the invention are administered in combination with alum. In another specific embodiment, Therapeutics of the invention are administered in combination with QS-21. Further adjuvants that may be administered with the Therapeutics of the invention include, but are not limited to, Monophosphoryl lipid immunomodulator, AdjuVax 100a, QS-21, QS-18, CRL1005, Aluminum salts, MF-59, and Virosomal adjuvant technology. Vaccines that may be administered with the Therapeutics of the invention include, but are not limited to, vaccines directed toward protection against MMR (measles, mumps, rubella), polio, varicella, tetanus/diptheria, hepatitis A, hepatitis B, haemophilus influenzae B, whooping cough, pneumonia, influenza, Lyme's Disease, rotavirus, cholera, yellow fever, Japanese encephalitis, poliomyelitis, rabies, typhoid fever, and pertussis. Combinations may be administered

either concomitantly, e.g., as an admixture, separately but simultaneously or concurrently; or sequentially. This includes presentations in which the combined agents are administered together as a therapeutic mixture, and also procedures in which the combined agents are administered separately but simultaneously, e.g., as through separate intravenous lines into the same individual. Administration "in combination" further includes the separate administration of one of the compounds or agents given first, followed by the second.

The Therapeutics of the invention may be administered alone or in combination with other therapeutic agents. Therapeutic agents that may be administered in combination with the Therapeutics of the invention, include but not limited to, other members of the TNF family, chemotherapeutic agents, antibiotics, steroidal and non-steroidal anti-inflammatories, conventional immunotherapeutic agents, cytokines and/or growth factors. Combinations may be administered either concomitantly, e.g., as an admixture, separately but simultaneously or concurrently; or sequentially. This includes presentations in which the combined agents are administered together as a therapeutic mixture, and also procedures in which the combined agents are administered separately but simultaneously, e.g., as through separate intravenous lines into the same individual. Administration "in combination" further includes the separate administration of one of the compounds or agents given first, followed by the second.

In one embodiment, the Therapeutics of the invention are administered in combination with members of the TNF family. TNF, TNF-related or TNF-like molecules that may be administered with the Therapeutics of the invention include, but are not limited to, soluble forms of TNF-alpha, lymphotoxin-alpha (LT-alpha, also known as TNF-beta), LT-beta (found in complex heterotrimer LT-alpha2-beta), OPGL, FasL, CD27L, CD30L, CD40L, 4-1BBL, DcR3, OX40L, TNF-gamma (International Publication No. WO 96/14328), AIM-I (International Publication No. WO 97/33899), endokine-alpha (International Publication No. WO 98/07880), TR6 (International Publication No. WO 98/30694), OPG, and neutrokine-alpha (International Publication No. WO 98/18921, OX40, and nerve growth factor (NGF), and soluble forms of Fas, CD30, CD27, CD40 and 4-IBB, TR2 (International Publication No. WO 96/34095), DR3 (International Publication No. WO 97/33904), DR4 (International Publication No.

WO 98/32856), TR5 (International Publication No. WO 98/30693), TR6 (International Publication No. WO 98/30694), TR7 (International Publication No. WO 98/41629), TRANK, TR9 (International Publication No. WO 98/56892),TR10 (International Publication No. WO 98/54202), 312C2 (International Publication No. WO 98/06842), and TR12, and soluble forms CD154, CD70, and CD153.

In certain embodiments, Therapeutics of the invention are administered in combination with antiretroviral agents, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and/or protease inhibitors. Nucleoside reverse transcriptase inhibitors that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, RETROVIR™ (zidovudine/AZT), VIDEX™ (didanosine/ddI), HIVID™ (zalcitabine/ddC), ZERIT™ (stavudine/d4T), EPIVIR™ (lamivudine/3TC), and COMBIVIR™ (zidovudine/lamivudine). Non-nucleoside reverse transcriptase inhibitors that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, VIRAMUNE™ (nevirapine), RESCRIPTOR™ (delavirdine), and SUSTIVA™ (efavirenz). Protease inhibitors that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, CRIXIVAN™ (indinavir), NORVIR™ (ritonavir), INVIRASE™ (saquinavir), and VIRACEPT™ (nelfinavir). In a specific embodiment, antiretroviral agents, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and/or protease inhibitors may be used in any combination with Therapeutics of the invention to treat AIDS and/or to prevent or treat HIV infection.

In other embodiments, Therapeutics of the invention may be administered in combination with anti-opportunistic infection agents. Anti-opportunistic agents that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, TRIMETHOPRIM-SULFAMETHOXAZOLE™, DAPSONE™, PENTAMIDINE™, ATOVAQUONE™, ISONIAZID™, RIFAMPIN™, PYRAZINAMIDE™, ETHAMBUTOL™, RIFABUTIN™, CLARITHROMYCIN™, AZITHROMYCIN™, GANCICLOVIR™, FOSCARNET™, CIDOFOVIR™, FLUCONAZOLE™, ITRACONAZOLE™, KETOCONAZOLE™, ACYCLOVIR™, FAMCICOLVIR™, PYRIMETHAMINE™, LEUCOVORIN™, NEUPOGEN™

(filgrastim/G-CSF), and LEUKINE™ (sargramostim/GM-CSF). In a specific embodiment, Therapeutics of the invention are used in any combination with TRIMETHOPRIM-SULFAMETHOXAZOLE™, DAPSONE™, PENTAMIDINE™, and/or ATOVAQUONE™ to prophylactically treat or prevent an opportunistic *Pneumocystis carinii* pneumonia infection. In another specific embodiment, Therapeutics of the invention are used in any combination with ISONIAZID™, RIFAMPIN™, PYRAZINAMIDE™, and/or ETHAMBUTOL™ to prophylactically treat or prevent an opportunistic *Mycobacterium avium* complex infection. In another specific embodiment, Therapeutics of the invention are used in any combination with RIFABUTIN™, CLARITHROMYCIN™, and/or AZITHROMYCIN™ to prophylactically treat or prevent an opportunistic *Mycobacterium tuberculosis* infection. In another specific embodiment, Therapeutics of the invention are used in any combination with GANCICLOVIR™, FOSCARNET™, and/or CIDOFOVIR™ to prophylactically treat or prevent an opportunistic cytomegalovirus infection. In another specific embodiment, Therapeutics of the invention are used in any combination with FLUCONAZOLE™, ITRACONAZOLE™, and/or KETOCONAZOLE™ to prophylactically treat or prevent an opportunistic fungal infection. In another specific embodiment, Therapeutics of the invention are used in any combination with ACYCLOVIR™ and/or FAMCICOLVIR™ to prophylactically treat or prevent an opportunistic herpes simplex virus type I and/or type II infection. In another specific embodiment, Therapeutics of the invention are used in any combination with PYRIMETHAMINE™ and/or LEUCOVORIN™ to prophylactically treat or prevent an opportunistic *Toxoplasma gondii* infection. In another specific embodiment, Therapeutics of the invention are used in any combination with LEUCOVORIN™ and/or NEUPOGEN™ to prophylactically treat or prevent an opportunistic bacterial infection.

In a further embodiment, the Therapeutics of the invention are administered in combination with an antiviral agent. Antiviral agents that may be administered with the Therapeutics of the invention include, but are not limited to, acyclovir, ribavirin, amantadine, and remantidine.

In a further embodiment, the Therapeutics of the invention are administered in combination with an antibiotic agent. Antibiotic agents that may be administered with the

Therapeutics of the invention include, but are not limited to, amoxicillin, beta-lactamases, aminoglycosides, beta-lactam (glycopeptide), beta-lactamases, Clindamycin, chloramphenicol, cephalosporins, ciprofloxacin, ciprofloxacin, erythromycin, fluoroquinolones, macrolides, metronidazole, penicillins, quinolones, rifampin, streptomycin, sulfonamide, tetracyclines, trimethoprim, trimethoprim-sulfamthoxazole, and vancomycin.

Conventional nonspecific immunosuppressive agents, that may be administered in combination with the Therapeutics of the invention include, but are not limited to, steroids, cyclosporine, cyclosporine analogs, cyclophosphamide methylprednisone, prednisone, azathioprine, FK-506, 15-deoxyspergualin, and other immunosuppressive agents that act by suppressing the function of responding T cells.

In specific embodiments, Therapeutics of the invention are administered in combination with immunosuppressants. Immunosuppressants preparations that may be administered with the Therapeutics of the invention include, but are not limited to, ORTHOCLONE™ (OKT3), SANDIMMUNE™/NEORAL™/SANGDYA™ (cyclosporin), PROGRAF™ (tacrolimus), CELLCEPT™ (mycophenolate), Azathioprine, glucorticosteroids, and RAPAMUNE™ (sirolimus). In a specific embodiment, immunosuppressants may be used to prevent rejection of organ or bone marrow transplantation.

In an additional embodiment, Therapeutics of the invention are administered alone or in combination with one or more intravenous immune globulin preparations. Intravenous immune globulin preparations that may be administered with the Therapeutics of the invention include, but not limited to, GAMMAR™, IVEEGAM™, SANDOGLOBULIN™, GAMMAGARD S/D™, and GAMIMUNE™. In a specific embodiment, Therapeutics of the invention are administered in combination with intravenous immune globulin preparations in transplantation therapy (e.g., bone marrow transplant).

In an additional embodiment, the Therapeutics of the invention are administered alone or in combination with an anti-inflammatory agent. Anti-inflammatory agents that may be administered with the Therapeutics of the invention include, but are not limited to, glucocorticoids and the nonsteroidal anti-inflammatories, aminoarylcarboxylic acid

derivatives, arylacetic acid derivatives, arylbutyric acid derivatives, arylcarboxylic acids, arylpropionic acid derivatives, pyrazoles, pyrazolones, salicylic acid derivatives, thiazinecarboxamides, e-acetamidocaproic acid, S-adenosylmethionine, 3-amino-4-hydroxybutyric acid, amixetrine, bendazac, benzydamine, bucolome, difenpiramide, ditazol, emorfazone, guaiazulene, nabumetone, nimesulide, orgotein, oxaceprol, paranyline, perisoxal, pifoxime, proquazone, proxazole, and tenidap.

In another embodiment, compostions of the invention are administered in combination with a chemotherapeutic agent. Chemotherapeutic agents that may be administered with the Therapeutics of the invention include, but are not limited to, antibiotic derivatives (e.g., doxorubicin, bleomycin, daunorubicin, and dactinomycin); antiestrogens (e.g., tamoxifen); antimetabolites (e.g., fluorouracil, 5-FU, methotrexate, floxuridine, interferon alpha-2b, glutamic acid, plicamycin, mercaptopurine, and 6-thioguanine); cytotoxic agents (e.g., carmustine, BCNU, lomustine, CCNU, cytosine arabinoside, cyclophosphamide, estramustine, hydroxyurea, procarbazine, mitomycin, busulfan, cis-platin, and vincristine sulfate); hormones (e.g., medroxyprogesterone, estramustine phosphate sodium, ethinyl estradiol, estradiol, megestrol acetate, methyltestosterone, diethylstilbestrol diphosphate, chlorotrianisene, and testolactone); nitrogen mustard derivatives (e.g., mephalen, chorambucil, mechlorethamine (nitrogen mustard) and thiotepa); steroids and combinations (e.g., bethamethasone sodium phosphate); and others (e.g., dicarbazine, asparaginase, mitotane, vincristine sulfate, vinblastine sulfate, and etoposide).

In a specific embodiment, Therapeutics of the invention are administered in combination with CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) or any combination of the components of CHOP. In another embodiment, Therapeutics of the invention are administered in combination with Rituximab. In a further embodiment, Therapeutics of the invention are administered with Rituxmab and CHOP, or Rituxmab and any combination of the components of CHOP.

In an additional embodiment, the Therapeutics of the invention are administered in combination with cytokines. Cytokines that may be administered with the Therapeutics of the invention include, but are not limited to, IL2, IL3, IL4, IL5, IL6, IL7, IL10, IL12, IL13, IL15, anti-CD40, CD40L, IFN-gamma and TNF-alpha. In

another embodiment, Therapeutics of the invention may be administered with any interleukin, including, but not limited to, IL-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, and IL-21.

In an additional embodiment, the Therapeutics of the invention are administered in combination with angiogenic proteins. Angiogenic proteins that may be administered with the Therapeutics of the invention include, but are not limited to, Glioma Derived Growth Factor (GDGF), as disclosed in European Patent Number EP-399816; Platelet Derived Growth Factor-A (PDGF-A), as disclosed in European Patent Number EP-682110; Platelet Derived Growth Factor-B (PDGF-B), as disclosed in European Patent Number EP-282317; Placental Growth Factor (PlGF), as disclosed in International Publication Number WO 92/06194; Placental Growth Factor-2 (PlGF-2), as disclosed in Hauser et al., Gorwth Factors, 4:259-268 (1993); Vascular Endothelial Growth Factor (VEGF), as disclosed in International Publication Number WO 90/13649; Vascular Endothelial Growth Factor-A (VEGF-A), as disclosed in European Patent Number EP-506477; Vascular Endothelial Growth Factor-2 (VEGF-2), as disclosed in International Publication Number WO 96/39515; Vascular Endothelial Growth Factor B (VEGF-3); Vascular Endothelial Growth Factor B-186 (VEGF-B186), as disclosed in International Publication Number WO 96/26736; Vascular Endothelial Growth Factor-D (VEGF-D), as disclosed in International Publication Number WO 98/02543; Vascular Endothelial Growth Factor-D (VEGF-D), as disclosed in International Publication Number WO 98/07832, and Vascular Endothelial Growth Factor-E (VEGF-E), as disclosed in German Patent Number DE19639601. The above mentioned references are incorporated herein by reference herein.

In an additional embodiment, the Therapeutics of the invention are administered in combination with hematopoietic growth factors. Hematopoietic growth factors that may be administered with the Therapeutics of the invention include, but are not limited to, LEUKINE™ (SARGRAMOSTIM™) and NEUPOGEN™ (FILGRASTIM™).

In an additional embodiment, the Therapeutics of the invention are administered in combination with Fibroblast Growth Factors. Fibroblast Growth Factors that may be administered with the Therapeutics of the invention include, but are not limited to, FGF-

1, FGF-2, FGF-3, FGF-4, FGF-5, FGF-6, FGF-7, FGF-8, FGF-9, FGF-10, FGF-11, FGF-12, FGF-13, FGF-14, and FGF-15.

In additional embodiments, the Therapeutics of the invention are administered in combination with other therapeutic or prophylactic regimens, such as, for example, radiation therapy.

Example 10: Method of Treating Decreased Levels of Follistatin-3.

The present invention relates to a method for treating an individual in need of a decreased level of follistatin-3 activity in the body comprising, administering to such an individual a composition comprising a therapeutically effective amount of follistatin-3 antagonist. Preferred antagonists for use in the present invention are follistatin-3-specific antibodies.

Moreover, it will be appreciated that conditions caused by a decrease in the standard or normal expression level of follistatin-3, in an individual can be treated by administering follistatin-3, preferably in the secreted form. Thus, the invention also provides a method of treatment of an individual in need of an increased level of follistatin-3 polypeptide comprising administering to such an individual a pharmaceutical composition comprising an amount of follistatin-3 to increase the activity level of follistatin-3 in such an individual.

For example, a patient with decreased levels of follistatin-3 polypeptide receives a daily dose 0.1-100 ug/kg of the polypeptide for six consecutive days. Preferably, the polypeptide is in the secreted form. The exact details of the dosing scheme, based on administration and formulation, are provided in Example 8.

Example 11: Method of Treating Increased Levels of Follistatin-3.

The present invention also relates to a method for treating an individual in need of an increased level of follistatin-3 activity in the body comprising administering to such an individual a composition comprising a therapeutically effective amount of follistatin-3 or an agonist thereof.

Antisense technology is used to inhibit production of follistatin-3. This technology is one example of a method of decreasing levels of follistatin-3 polypeptide,

preferably a secreted form, due to a variety of etiologies, such as cancer.

For example, a patient diagnosed with abnormally increased levels of follistatin-3 is administered intravenously antisense polynucleotides at 0.5, 1.0, 1.5, 2.0 and 3.0 mg/kg day for 21 days. This treatment is repeated after a 7-day rest period if the treatment was well tolerated. The formulation of the antisense polynucleotide is provided in Example 8.

Example 12: Method of Treatment Using Gene Therapy - Ex Vivo.

One method of gene therapy transplants fibroblasts, which are capable of expressing follistatin-3 polypeptides, onto a patient. Generally, fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin) is added. The flasks are then incubated at 37 degree C for approximately one week.

At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

pMV-7 (Kirschmeier, P.T. et al., DNA, 7:219-25 (1988)), flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding follistatin-3 can be amplified using PCR primers which correspond to the 5' and 3' end sequences respectively as set forth in Example 1. Preferably, the 5' primer contains an EcoRI site and the 3' primer includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two

fragments. The ligation mixture is then used to transform bacteria HB101, which are then plated onto agar containing kanamycin for the purpose of confirming that the vector contains properly inserted follistatin-3.

The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the follistatin-3 gene is then added to the media and the packaging cells transduced with the vector. The packaging cells now produce infectious viral particles containing the follistatin-3 gene(the packaging cells are now referred to as producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his. Once the fibroblasts have been efficiently infected, the fibroblasts are analyzed to determine whether follistatin-3 protein is produced.

The engineered fibroblasts are then transplanted onto the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads.

Example 13: Gene Therapy Using Endogenous Follistatin-3 Gene.

Another method of gene therapy according to the present invention involves operably associating the endogenous follistatin-3 sequence with a promoter via homologous recombination as described, for example, in U.S. Patent No. 5,641,670, issued June 24, 1997; International Publication No. WO 96/29411, published September 26, 1996; International Publication No. WO 94/12650, published August 4, 1994; Koller et al., Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); and Zijlstra et al., Nature 342:435-438 (1989). This method involves the activation of a gene which is present in

the target cells, but which is not expressed in the cells, or is expressed at a lower level than desired.

Polynucleotide constructs are made which contain a promoter and targeting sequences, which are homologous to the 5' non-coding sequence of endogenous follistatin-3, flanking the promoter. The targeting sequence will be sufficiently near the 5' end of follistatin-3 so the promoter will be operably linked to the endogenous sequence upon homologous recombination. The promoter and the targeting sequences can be amplified using PCR. Preferably, the amplified promoter contains distinct restriction enzyme sites on the 5' and 3' ends. Preferably, the 3' end of the first targeting sequence contains the same restriction enzyme site as the 5' end of the amplified promoter and the 5' end of the second targeting sequence contains the same restriction site as the 3' end of the amplified promoter.

The amplified promoter and the amplified targeting sequences are digested with the appropriate restriction enzymes and subsequently treated with calf intestinal phosphatase. The digested promoter and digested targeting sequences are added together in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The construct is size fractionated on an agarose gel then purified by phenol extraction and ethanol precipitation.

In this Example, the polynucleotide constructs are administered as naked polynucleotides via electroporation. However, the polynucleotide constructs may also be administered with transfection-facilitating agents, such as liposomes, viral sequences, viral particles, precipitating agents, etc. Such methods of delivery are known in the art.

Once the cells are transfected, homologous recombination will take place which results in the promoter being operably linked to the endogenous follistatin-3 sequence. This results in the expression of follistatin-3 in the cell. Expression may be detected by immunological staining, or any other method known in the art.

Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in DMEM + 10% fetal calf serum. Exponentially growing or early stationary phase fibroblasts are trypsinized and rinsed from the plastic surface with nutrient medium. An aliquot of the cell suspension is removed for counting, and the remaining

cells are subjected to centrifugation. The supernatant is aspirated and the pellet is resuspended in 5 ml of electroporation buffer (20 mM HEPES pH 7.3, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose). The cells are recentrifuged, the supernatant aspirated, and the cells resuspended in electroporation buffer containing 1 mg/ml acetylated bovine serum albumin. The final cell suspension contains approximately $3X10^6$ cells/ml. Electroporation should be performed immediately following resuspension.

Plasmid DNA is prepared according to standard techniques. For example, to construct a plasmid for targeting to the follistatin-3 locus, plasmid pUC18 (MBI Fermentas, Amherst, NY) is digested with HindIII. The CMV promoter is amplified by PCR with an XbaI site on the 5' end and a BamHI site on the 3'end. Two follistatin-3 non-coding sequences are amplified via PCR: one follistatin-3 non-coding sequence follistatin-3 fragment 1) is amplified with a HindIII site at the 5' end and an Xba site at the 3'end; the other follistatin-3 non-coding sequence (follistatin-3 fragment 2) is amplified with a BamHI site at the 5'end and a HindIII site at the 3'end. The CMV promoter and follistatin-3 fragments are digested with the appropriate enzymes (CMV promoter - XbaI and BamHI; follistatin-3 fragment 1 - XbaI; follistatin-3 fragment 2 - BamHI) and ligated together. The resulting ligation product is digested with HindIII, and ligated with the HindIII-digested pUC18 plasmid

Plasmid DNA is added to a sterile cuvette with a 0.4 cm electrode gap (Bio-Rad). The final DNA concentration is generally at least 120 µg/ml. 0.5 ml of the cell suspension (containing approximately $1.5.X10^6$ cells) is then added to the cuvette, and the cell suspension and DNA solutions are gently mixed. Electroporation is performed with a Gene-Pulser apparatus (Bio-Rad). Capacitance and voltage are set at 960 µF and 250-300 V, respectively. As voltage increases, cell survival decreases, but the percentage of surviving cells that stably incorporate the introduced DNA into their genome increases dramatically. Given these parameters, a pulse time of approximately 14-20 mSec should be observed.

Electroporated cells are maintained at room temperature for approximately 5 min, and the contents of the cuvette are then gently removed with a sterile transfer pipette. The cells are added directly to 10 ml of prewarmed nutrient media (DMEM with

15% calf serum) in a 10 cm dish and incubated at 37 degree C. The following day, the media is aspirated and replaced with 10 ml of fresh media and incubated for a further 16-24 hours.

The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product. The fibroblasts can then be introduced into a patient as described above.

Example 14: Method of Treatment Using Gene Therapy - In Vivo.

Another aspect of the present invention is using in vivo gene therapy methods to treat disorders, diseases and conditions. The gene therapy method relates to the introduction of naked nucleic acid (DNA, RNA, and antisense DNA or RNA) follistatin-3 sequences into an animal to increase or decrease the expression of the follistatin-3 polypeptide. The follistatin-3 polynucleotide may be operatively linked to a promoter or any other genetic elements necessary for the expression of the follistatin-3 polypeptide by the target tissue. Such gene therapy and delivery techniques and methods are known in the art, see, for example, WO90/11092, WO98/11779; U.S. Patent NO. 5693622, 5705151, 5580859; Tabata H et al. (1997) Cardiovasc. Res. 35(3):470-479, Chao J et al. (1997) Pharmacol. Res. 35(6):517-522, Wolff J.A. (1997) Neuromuscul. Disord. 7(5):314-318, Schwartz B. et al. (1996) Gene Ther. 3(5):405-411, Tsurumi Y. et al. (1996) Circulation 94(12):3281-3290 (incorporated herein by reference).

The follistatin-3 polynucleotide constructs may be delivered by any method that delivers injectable materials to the cells of an animal, such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, intestine and the like). The follistatin-3 polynucleotide constructs can be delivered in a pharmaceutically acceptable liquid or aqueous carrier.

The term "naked" polynucleotide, DNA or RNA, refers to sequences that are free from any delivery vehicle that acts to assist, promote, or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. However, the follistatin-3 polynucleotides may also be delivered in liposome formulations (such as those taught in Felgner P.L. et al. (1995)

Ann. NY Acad. Sci. 772:126-139 and Abdallah B. et al. (1995) Biol. Cell 85(1):1-7) which can be prepared by methods well known to those skilled in the art.

The follistatin-3 polynucleotide vector constructs used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will they contain sequences that allow for replication. Any strong promoter known to those skilled in the art can be used for driving the expression of DNA. Unlike other gene therapies techniques, one major advantage of introducing naked nucleic acid sequences into target cells is the transitory nature of the polynucleotide synthesis in the cells. Studies have shown that non-replicating DNA sequences can be introduced into cells to provide production of the desired polypeptide for periods of up to six months.

The follistatin-3 polynucleotide construct can be delivered to the interstitial space of tissues within the an animal, including of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. Delivery to the interstitial space of muscle tissue is preferred for the reasons discussed below. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression may be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts. In vivo muscle cells are particularly competent in their ability to take up and express polynucleotides.

For the naked follistatin-3 polynucleotide injection, an effective dosage amount of DNA or RNA will be in the range of from about 0.05 g/kg body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site

of injection. The appropriate and effective dosage of nucleic acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration. The preferred route of administration is by the parenteral route of injection into the interstitial space of tissues. However, other parenteral routes may also be used, such as, inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or mucous membranes of the nose. In addition, naked follistatin-3 polynucleotide constructs can be delivered to arteries during angioplasty by the catheter used in the procedure.

The dose response effects of injected follistatin-3 polynucleotide in muscle in vivo is determined as follows. Suitable follistatin-3 template DNA for production of mRNA coding for follistatin-3 polypeptide is prepared in accordance with a standard recombinant DNA methodology. The template DNA, which may be either circular or linear, is either used as naked DNA or complexed with liposomes. The quadriceps muscles of mice are then injected with various amounts of the template DNA.

Five to six week old female and male Balb/C mice are anesthetized by intraperitoneal injection with 0.3 ml of 2.5% Avertin. A 1.5 cm incision is made on the anterior thigh, and the quadriceps muscle is directly visualized. The follistatin-3 template DNA is injected in 0.1 ml of carrier in a 1 cc syringe through a 27 gauge needle over one minute, approximately 0.5 cm from the distal insertion site of the muscle into the knee and about 0.2 cm deep. A suture is placed over the injection site for future localization, and the skin is closed with stainless steel clips.

After an appropriate incubation time (e.g., 7 days) muscle extracts are prepared by excising the entire quadriceps. Every fifth 15 um cross-section of the individual quadriceps muscles is histochemically stained for follistatin-3 protein expression. A time course for follistatin-3 protein expression may be done in a similar fashion except that quadriceps from different mice are harvested at different times. Persistence of follistatin-3 DNA in muscle following injection may be determined by Southern blot analysis after preparing total cellular DNA and HIRT supernatants from injected and control mice. The results of the above experimentation in mice can be use to extrapolate proper dosages and other treatment parameters in humans and other animals using follistatin-3 naked DNA.

Example 15: Follistatin-3 Transgenic Animals.

The follistatin-3 polypeptides can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, hamsters, guinea pigs, pigs, micro-pigs, goats, sheep, cows and non-human primates, e.g., baboons, monkeys, and chimpanzees may be used to generate transgenic animals. In a specific embodiment, techniques described herein or otherwise known in the art, are used to express polypeptides of the invention in humans, as part of a gene therapy protocol.

Any technique known in the art may be used to introduce the transgene (i.e., polynucleotides of the invention) into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Paterson et al., Appl. Microbiol. Biotechnol. 40:691-698 (1994); Carver et al., Biotechnology (NY) 11:1263-1270 (1993); Wright et al., Biotechnology (NY) 9:830-834 (1991); and Hoppe et al., U.S. Pat. No. 4,873,191 (1989)); retrovirus mediated gene transfer into germ lines (Van der Putten et al., Proc. Natl. Acad. Sci., USA 82:6148-6152 (1985)), blastocysts or embryos; gene targeting in embryonic stem cells (Thompson et al., Cell 56:313-321 (1989)); electroporation of cells or embryos (Lo, 1983, Mol Cell. Biol. 3:1803-1814 (1983)); introduction of the polynucleotides of the invention using a gene gun (see, e.g., Ulmer et al., Science 259:1745 (1993); introducing nucleic acid constructs into embryonic pleuripotent stem cells and transferring the stem cells back into the blastocyst; and sperm-mediated gene transfer (Lavitrano et al., Cell 57:717-723 (1989); etc. For a review of such techniques, see Gordon, "Transgenic Animals," Intl. Rev. Cytol. 115:171-229 (1989), which is incorporated by reference herein in its entirety.

Any technique known in the art may be used to produce transgenic clones containing polynucleotides of the invention, for example, nuclear transfer into enucleated oocytes of nuclei from cultured embryonic, fetal, or adult cells induced to quiescence (Campell et al., Nature 380:64-66 (1996); Wilmut et al., Nature 385:810-813 (1997)).

The present invention provides for transgenic animals that carry the transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, i.e., mosaic animals or chimeric. The transgene may be integrated as a single transgene

or as multiple copies such as in concatamers, e.g., head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (Lasko et al., Proc. Natl. Acad. Sci. USA 89:6232-6236 (1992)). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the polynucleotide transgene be integrated into the chromosomal site of the endogenous gene, gene targeting is preferred.

Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous gene in only that cell type, by following, for example, the teaching of Gu et al. (Gu et al., Science 265:103-106 (1994)). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. The contents of each of the documents recited in this paragraph is herein incorporated by reference in its entirety.

Once transgenic animals have been generated, the expression of the recombinant gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to verify that integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, in situ hybridization analysis, and reverse transcriptase-PCR (rt-PCR). Samples of transgenic gene-expressing tissue may also be evaluated immunocytochemically or immunohistochemically using antibodies specific for the transgene product.

Once the founder animals are produced, they may be bred, inbred, outbred, or crossbred to produce colonies of the particular animal. Examples of such breeding strategies include, but are not limited to: outbreeding of founder animals with more than

one integration site in order to establish separate lines; inbreeding of separate lines in order to produce compound transgenics that express the transgene at higher levels because of the effects of additive expression of each transgene; crossing of heterozygous transgenic animals to produce animals homozygous for a given integration site in order to both augment expression and eliminate the need for screening of animals by DNA analysis; crossing of separate homozygous lines to produce compound heterozygous or homozygous lines; and breeding to place the transgene on a distinct background that is appropriate for an experimental model of interest.

Transgenic animals of the invention have uses which include, but are not limited to, animal model systems useful in elaborating the biological function of follistatin-3 polypeptides, studying conditions and/or disorders associated with aberrant follistatin-3 expression, and in screening for compounds effective in ameliorating such conditions and/or disorders

Example 16: Follistatin-3 Knock-Out Animals.

Endogenous follistatin-3 gene expression can also be reduced by inactivating or "knocking out" the follistatin-3 gene and/or its promoter using targeted homologous recombination. (E.g., see Smithies et al., Nature 317:230-234 (1985); Thomas & Capecchi, Cell 51:503-512 (1987); Thompson et al., Cell 5:313-321 (1989); each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional polynucleotide of the invention (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous polynucleotide sequence (either the coding regions or regulatory regions of the gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express polypeptides of the invention in vivo. In another embodiment, techniques known in the art are used to generate knockouts in cells that contain, but do not express the gene of interest. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the targeted gene. Such approaches are particularly suited in research and agricultural fields where modifications to embryonic stem cells can be used to generate animal offspring with an inactive targeted gene (e.g., see Thomas & Capecchi 1987 and Thompson 1989, supra). However this approach can be routinely

adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site in vivo using appropriate viral vectors that will be apparent to those of skill in the art.

In further embodiments of the invention, cells that are genetically engineered to express the polypeptides of the invention, or alternatively, that are genetically engineered not to express the polypeptides of the invention (e.g., knockouts) are administered to a patient in vivo. Such cells may be obtained from the patient (i.e., animal, including human) or an MHC compatible donor and can include, but are not limited to fibroblasts, bone marrow cells, blood cells (e.g., lymphocytes), adipocytes, muscle cells, endothelial cells etc. The cells are genetically engineered in vitro using recombinant DNA techniques to introduce the coding sequence of polypeptides of the invention into the cells, or alternatively, to disrupt the coding sequence and/or endogenous regulatory sequence associated with the polypeptides of the invention, e.g., by transduction (using viral vectors, and preferably vectors that integrate the transgene into the cell genome) or transfection procedures, including, but not limited to, the use of plasmids, cosmids, YACs, naked DNA, electroporation, liposomes, etc. The coding sequence of the polypeptides of the invention can be placed under the control of a strong constitutive or inducible promoter or promoter/enhancer to achieve expression, and preferably secretion, of the follistatin-3 polypeptides. The engineered cells which express and preferably secrete the polypeptides of the invention can be introduced into the patient systemically, e.g., in the circulation, or intraperitoneally.

Alternatively, the cells can be incorporated into a matrix and implanted in the body, e.g., genetically engineered fibroblasts can be implanted as part of a skin graft, genetically engineered endothelial cells can be implanted as part of a lymphatic or vascular graft. (See, for example, Anderson et al. U.S. Patent No. 5,399,349; and Mulligan & Wilson, U.S. Patent No. 5,460,959 each of which is incorporated by reference herein in its entirety).

When the cells to be administered are non-autologous or non-MHC compatible cells, they can be administered using well known techniques which prevent the development of a host immune response against the introduced cells. For example, the cells may be introduced in an encapsulated form which, while allowing for an exchange

of components with the immediate extracellular environment, does not allow the introduced cells to be recognized by the host immune system.

Knock-out animals of the invention have uses which include, but are not limited to, animal model systems useful in elaborating the biological function of follistatin-3 polypeptides, studying conditions and/or disorders associated with aberrant follistatin-3 expression, and in screening for compounds effective in ameliorating such conditions and/or disorders.

Example 17: Assays Detecting Stimulation or Inhibition of B cell Proliferation and Differentiation.

Generation of functional humoral immune responses requires both soluble and cognate signaling between B-lineage cells and their microenvironment. Signals may impart a positive stimulus that allows a B-lineage cell to continue its programmed development, or a negative stimulus that instructs the cell to arrest its current developmental pathway. To date, numerous stimulatory and inhibitory signals have been found to influence B cell responsiveness including IL-2, IL-4, IL-5, IL-6, IL-7, IL10, IL-13, IL-14 and IL-15. Interestingly, these signals are by themselves weak effectors but can, in combination with various co-stimulatory proteins, induce activation, proliferation, differentiation, homing, tolerance and death among B cell populations.

One of the best studied classes of B-cell co-stimulatory proteins is the TNF-superfamily. Within this family CD40, CD27, and CD30 along with their respective ligands CD154, CD70, and CD153 have been found to regulate a variety of immune responses. Assays which allow for the detection and/or observation of the proliferation and differentiation of these B-cell populations and their precursors are valuable tools in determining the effects various proteins may have on these B-cell populations in terms of proliferation and differentiation. Listed below are two assays designed to allow for the detection of the differentiation, proliferation, or inhibition of B-cell populations and their precursors.

In Vitro Assay- Purified follistatin-3 protein, or truncated forms thereof, is assessed for its ability to induce activation, proliferation, differentiation or inhibition and/or death in B-cell populations and their precursors. The activity of follistatin-3

protein on purified human tonsillar B cells, measured qualitatively over the dose range from 0.1 to 10,000 ng/mL, is assessed in a standard B-lymphocyte co-stimulation assay in which purified tonsillar B cells are cultured in the presence of either formalin-fixed Staphylococcus aureus Cowan I (SAC) or immobilized anti-human IgM antibody as the priming agent. Second signals such as IL-2 and IL-15 synergize with SAC and IgM crosslinking to elicit B cell proliferation as measured by tritiated-thymidine incorporation. Novel synergizing agents can be readily identified using this assay. The assay involves isolating human tonsillar B cells by magnetic bead (MACS) depletion of CD3-positive cells. The resulting cell population is greater than 95% B cells as assessed by expression of CD45R(B220).

Various dilutions of each sample are placed into individual wells of a 96-well plate to which are added 10^5 B-cells suspended in culture medium (RPMI 1640 containing 10% FBS, 5 X 10^{-5}M 2ME, 100U/ml penicillin, 10ug/ml streptomycin, and 10^{-5} dilution of SAC) in a total volume of 150ul. Proliferation or inhibition is quantitated by a 20h pulse (1uCi/well) with 3H-thymidine (6.7 Ci/mM) beginning 72h post factor addition. The positive and negative controls are IL2 and medium respectively.

In Vivo Assay- BALB/c mice are injected (i.p.) twice per day with buffer only, or 2 mg/Kg of follistatin-3 protein, or truncated forms thereof. Mice receive this treatment for 4 consecutive days, at which time they are sacrificed and various tissues and serum collected for analyses. Comparison of H&E sections from normal and follistatin-3 protein-treated spleens identify the results of the activity of follistatin-3 protein on spleen cells, such as the diffusion of peri-arterial lymphatic sheaths, and/or significant increases in the nucleated cellularity of the red pulp regions, which may indicate the activation of the differentiation and proliferation of B-cell populations. Immunohistochemical studies using a B cell marker, anti-CD45R(B220), are used to determine whether any physiological changes to splenic cells, such as splenic disorganization, are due to increased B-cell representation within loosely defined B-cell zones that infiltrate established T-cell regions

Flow cytometric analyses of the spleens from follistatin-3 protein-treated mice is used to indicate whether follistatin-3 protein specifically increases the proportion of ThB+, CD45R(B220)dull B cells over that which is observed in control mice.

Likewise, a predicted consequence of increased mature B-cell representation in vivo is a relative increase in serum Ig titers. Accordingly, serum IgM and IgA levels are compared between buffer and follistatin-3 protein-treated mice.

The studies described in this example tested activity in follistatin-3 protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of follistatin-3 polynucleotides (e.g., gene therapy), agonists, and/or antagonists of follistatin-3.

Example 18: T Cell Proliferation Assay.

A CD3-induced proliferation assay is performed on PBMCs and is measured by the uptake of ^{3}H-thymidine. The assay is performed as follows. Ninety-six well plates are coated with 100 microliters/well of mAb to CD3 (HIT3a, Pharmingen) or isotype-matched control mAb (B33.1) overnight at 4°C (1 micrograms/ml in .05M bicarbonate buffer, pH 9.5), then washed three times with PBS. PBMC are isolated by F/H gradient centrifugation from human peripheral blood and added to quadruplicate wells (5 x 10^4/well) of mAb coated plates in RPMI containing 10% FCS and P/S in the presence of varying concentrations of follistatin-3 protein (total volume 200 microliters). Relevant protein buffer and medium alone are controls. After 48 hr. culture at 37°C, plates are spun for 2 min. at 1000 rpm and 100 microliters of supernatant is removed and stored -20°C for measurement of IL-2 (or other cytokines) if effect on proliferation is observed. Wells are supplemented with 100 microliters of medium containing 0.5 microCi of ^{3}H-thymidine and cultured at 37°C for 18-24 hr. Wells are harvested and incorporation of ^{3}H-thymidine used as a measure of proliferation. Anti-CD3 alone is the positive control for proliferation. IL-2 (100 U/ml) is also used as a control which enhances proliferation. Control antibody which does not induce proliferation of T cells is used as the negative controls for the effects of follistatin-3 proteins.

The studies described in this example tested activity in follistatin-3 protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of follistatin-3 polynucleotides (e.g., gene therapy), agonists, and/or antagonists of follistatin-3.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

The entire disclosure of all publications (including patents, patent applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference.

Further, each of the Sequence Listings submitted herewith and with U.S. Provisional Application Serial No. 60/144,088, filed July 16, 1999, to which the present application claims benefit of priority, in both computer and paper forms, is hereby incorporated by reference in its entirety.

Applicant's or agent's file reference number	PF388PCT	International application No	UNASSIGNED

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 4, line 12.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution: American Type Culture Collection

Address of depositary institution (including postal code and country):
10801 University Boulevard
Manassas, Virginia 20110-2209
United States of America

Date of deposit	Accession Number
6 August 1997	209199

C. ADDITIONAL INDICATIONS (leave blank if not applicable) — This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

Europe
In respect to those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28 (4) EPC).

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

For receiving Office use only

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ATCC Deposit No. 209199

CANADA

The applicant requests that, until either a Canadian patent has been issued on the basis of an application or the application has been refused, or is abandoned and no longer subject to reinstatement, or is withdrawn, the Commissioner of Patents only authorizes the furnishing of a sample of the deposited biological material referred to in the application to an independent expert nominated by the Commissioner, the applicant must, by a written statement, inform the International Bureau accordingly before completion of technical preparations for publication of the international application.

NORWAY

The applicant hereby requests that the application has been laid open to public inspection (by the Norwegian Patent Office), or has been finally decided upon by the Norwegian Patent Office without having been laid open inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Norwegian Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Norwegian Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on the list of recognized experts drawn up by the Norwegian Patent Office or any person approved by the applicant in the individual case..

AUSTRALIA

The applicant hereby gives notice that the furnishing of a sample of a microorganism shall only be effected prior to the grant of a patent, or prior to the lapsing, refusal or withdrawal of the application, to a person who is a skilled addressee without an interest in the invention (Regulation 3.25(3) of the Australian Patents Regulations).

FINLAND

The applicant hereby requests that, until the application has been laid open to public inspection (by the National Board of Patents and Regulations), or has been finally decided upon by the National Board of Patents and Registration without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art.

UNITED KINGDOM

The applicant hereby requests that the furnishing of a sample of a microorganism shall only be made available to an expert. The request to this effect must be filed by the applicant with the International Bureau before the completion of the technical preparations for the international publication of the application.

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DENMARK

The applicant hereby requests that, until the application has been laid open to public inspection (by the Danish Patent Office), or has been finally decided upon by the Danish Patent office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Danish Patent Office not later that at the time when the application is made available to the public under Sections 22 and 33(3) of the Danish Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Danish Patent Office or any person by the applicant in the individual case.

SWEDEN

The applicant hereby requests that, until the application has been laid open to public inspection (by the Swedish Patent Office), or has been finally decided upon by the Swedish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the International Bureau before the expiration of 16 months from the priority date (preferably on the Form PCT/RO/134 reproduced in annex Z of Volume I of the PCT Applicant's Guide). If such a request has been filed by the applicant any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Swedish Patent Office or any person approved by a applicant in the individual case.

NETHERLANDS

The applicant hereby requests that until the date of a grant of a Netherlands patent or until the date on which the application is refused or withdrawn or lapsed, the microorganism shall be made available as provided in the 31F(1) of the Patent Rules only by the issue of a sample to an expert. The request to this effect must be furnished by the applicant with the Netherlands Industrial Property Office before the date on which the application is made available to the public under Section 22C or Section 25 of the Patents Act of the Kingdom of the Netherlands, whichever of the two dates occurs earlier.

What Is Claimed Is:

1. An isolated nucleic acid molecule nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:

(a) a nucleotide sequence encoding the follistatin-3 polypeptide having the complete amino acid sequence in SEQ ID NO:2 (i.e., positions -26 to 237 of SEQ ID NO:2);

(b) a nucleotide sequence encoding the follistatin-3 polypeptide having the complete amino acid sequence in SEQ ID NO:2 excepting the N-terminal methionine (i.e., positions -25 to 237 of SEQ ID NO:2);

(c) a nucleotide sequence encoding the predicted mature follistatin-3 polypeptide having the amino acid sequence at positions 1 to 237 in SEQ ID NO:2;

(d) a nucleotide sequence encoding the follistatin-3 polypeptide having the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209199;

(e) a nucleotide sequence encoding the follistatin-3 polypeptide having the complete amino acid sequence excepting the N-terminal methionine encoded by the cDNA clone contained in ATCC Deposit No. 209199;

(f) a nucleotide sequence encoding the mature follistatin-3 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209199; and

(g) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e) or (f) above.

2. The nucleic acid molecule of claim 1 wherein said polynucleotide has the complete nucleotide sequence in Figures 1A, 1B, and 1C (SEQ ID NO:1).

3. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence in Figures 1A, 1B, and 1C (SEQ ID NO:1) encoding the follistatin-3 polypeptide having the amino acid sequence in positions -26 to 237 of SEQ ID NO:2.

4. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence in Figures 1A, 1B, and 1C (SEQ ID NO:1) encoding the follistatin-3 polypeptide having the amino acid sequence in positions -25 to 237 of SEQ ID NO:2.

5. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence in Figures 1A, 1B, and 1C (SEQ ID NO:1) encoding the mature follistatin-3 polypeptide having the amino acid sequence from about 1 to about 237 in SEQ ID NO:2.

6. An isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:

(a) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence of residues n-237 of SEQ ID NO:2, where n is an integer in the range of -26-12;

(b) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence of residues -26-m of SEQ ID NO:2, where m is an integer in the range of -26-m of 207 to 237;

(c) a nucleotide sequence encoding a polypeptide having the amino acid sequence consisting of residues n-m of SEQ ID NO:2, where n and m are integers as defined respectively in (a) and (b) above, and

(d) a nucleotide sequence encoding a polypeptide consisting of a portion of the complete follistatin-3 amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209199 wherein said portion excludes from 1 to about 37 amino acids from the amino terminus of said complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209199;

(e) a nucleotide sequence encoding a polypeptide consisting of a portion of the complete follistatin-3 amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209199 wherein said portion excludes from 1 to about 20 amino acids from the carboxy terminus of said complete amino acid sequence encoded by the

cDNA clone contained in ATCC Deposit No. 209199; and

(f) a nucleotide sequence encoding a polypeptide consisting of a portion of the complete follistatin-3 amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209199 wherein said portion include a combination of any of the amino terminal and carboxy terminal deletions in (d) and (e), above.

7. The nucleic acid molecule of claim 1 wherein said polynucleotide has the complete nucleotide sequence of the cDNA clone contained in ATCC Deposit No. 209199.

8. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence encoding the follistatin-3 polypeptide having the complete amino acid sequence excepting the N-terminal methionine encoded by the cDNA clone contained in ATCC Deposit No. 209199

9 The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence encoding the mature follistatin-3 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209199.

10. An isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide having a nucleotide sequence identical to a nucleotide sequence in (a), (b), (c), (d), (e), (f), or (g) of claim 1 wherein said polynucleotide which hybridizes does not hybridize under stringent hybridization conditions to a polynucleotide having a nucleotide sequence consisting of only A residues or of only T residues.

11. An isolated nucleic acid molecule comprising a polynucleotide which encodes the amino acid sequence of an epitope-bearing portion of a follistatin-3 polypeptide having an amino acid sequence in (a), (b), (c), (d), (e), or (f) of claim 1.

12. The isolated nucleic acid molecule of claim 11, which encodes an

epitope-bearing portion of a follistatin-3 polypeptide wherein the amino acid sequence of said portion is selected from the group of sequences in SEQ ID NO:2 consisting of: about about Leu-14 to about Ala-20, from about Ser-46 to about Ile-55, from about Gly-88 to about Pro-97, from about Gly-113 to about Leu-133, from about Arg-138 to about Glu-146, from about Pro-177 to about Thr-191, and from about Gly-219 to about Val-237.

13. A method for making a recombinant vector comprising inserting an isolated nucleic acid molecule of claim 1 into a vector.

14. A recombinant vector produced by the method of claim 13.

15. A method of making a recombinant host cell comprising introducing the recombinant vector of claim 14 into a host cell.

16. A recombinant host cell produced by the method of claim 15.

17. A recombinant method for producing a follistatin-3 polypeptide, comprising culturing the recombinant host cell of claim 16 under conditions such that said polypeptide is expressed and recovering said polypeptide.

18. An isolated follistatin-3 polypeptide comprising an amino acid sequence at least 95% identical to a sequence selected from the group consisting of:

(a) the amino acid sequence of the full-length follistatin-3 polypeptide having the complete amino acid sequence shown in SEQ ID NO:2 (i.e., positions -26 to 237 of SEQ ID NO:2);

(b) the amino acid sequence of the full-length follistatin-3 polypeptide having the complete amino acid sequence shown in SEQ ID NO:2 excepting the N-terminal methionine (i.e., positions -25 to 237 of SEQ ID NO:2),

(c) the amino acid sequence of the predicted mature follistatin-3 polypeptide having the amino acid sequence at positions 1 to 237 in SEQ ID NO:2.

(d) the amino acid sequence of the full-length follistatin-3 polypeptide having the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209199;

(e) the amino acid sequence of the full-length follistatin-3 polypeptide having the complete amino acid sequence excepting the N-terminal methionine encoded by the cDNA clone contained in ATCC Deposit No. 209199; and

(f) the amino acid sequence of the mature follistatin-3 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209199.

19. An isolated polypeptide comprising an epitope-bearing portion of the follistatin-3 protein, wherein said portion is selected from the group consisting of: a polypeptide comprising amino acid residues from about Leu-14 to about Ala-20 of SEQ ID NO:2; a polypeptide comprising amino acid residues from about Ser-46 to about Ile-55 of SEQ ID NO:2; a polypeptide comprising amino acid residues from about Gly-88 to about Pro-97 of SEQ ID NO:2; a polypeptide comprising amino acid residues from about Gly-113 to about Leu-133 of SEQ ID NO:2; a polypeptide comprising amino acid residues from about Arg-138 to about Glu-146 of SEQ ID NO:2; a polypeptide comprising amino acid residues from about Pro-177 to about Thr-191 of SEQ ID NO:2; and a polypeptide comprising amino acid residues from about Gly-219 to about Val-237 of SEQ ID NO:2.

20. An isolated antibody that binds specifically to a follistatin-3 polypeptide of claim 18.

21. An isolated nucleic acid molecule comprising a polynucleotide having a sequence at least 95% identical to a sequence selected from the group consisting of:

(a) the nucleotide sequence of SEQ ID NO:4;

(b) the nucleotide sequence of SEQ ID NO:5;

(c) the nucleotide sequence of SEQ ID NO:6;

(d) the nucleotide sequence of SEQ ID NO:7;

(e) the nucleotide sequence of SEQ ID NO:8,

(f) the nucleotide sequence of SEQ ID NO:9,

(g) the nucleotide sequence of SEQ ID NO:10,

(h) the nucleotide sequence of SEQ ID NO:11;

(i) the nucleotide sequence of a portion of the sequence shown in Figures 1A, 1B, and 1C (SEQ ID NO:1) wherein said portion comprises at least 50 contiguous nucleotides from nucleotide 1 to 500; and

(j) the nucleotide sequence of a portion of the sequence shown in Figures 1A, 1B, and 1C (SEQ ID NO:1) wherein said portion consists of nucleotides 100-500, 200-500, 300-500, 400-500, 100-400, 200-400, 300-400, 100-300, 200-300, 100-200, 100-2495, 250-2495, 500-2495, 1000-2495, 1500-2495, 2000-2495, 100-2000, 250-2000, 500-2000, 1000-2000, 1500-2000, 100-1500, 250-1500, 500-1500, 1000-1500, 100-1000, 250-1000, and 500-1000 of SEQ ID NO.1; and

(k) a nucleotide sequence complementary to any of the nucleotide sequences in (a) through (j) above

1/5

Follistatin-3

```
   1 GCCGTCTCTGCGTTCGCCATGGATCCCGGGGCGCCAGGGCCACTCTGGCCTCTGCCCTGG   60
   1                 M  R  P  G  A  P  G  P  L  W  P  L  P  W      14

  61 GGGGCCCTGGCTTGGGCGTGGGCTTCGTGAGCTCCATGGGCTCGGGGAACCCCGCGCCC   120
  15 G  A  L  A  W  A  V  G  F  V  S  S  M  G  S  G  N  P  A  P    34

 121 GGTGGTGTTTGCTGGCTCCAGCAGGGCCAGGAGGCCACCTGCAGCCTGGTGCTCCAGACT   180
  35 G  G  V  C  W  L  Q  Q  G  Q  E  A  T  C  S  L  V  L  Q  T    54
        *                                                   #

 181 GATGTCACCCGGGCCGAGTGCTGTGCCTCCGGCAACATTGACACCGCCTGGTCCAACCTC   240
  55 D  V  T  R  A  E  C  C  A  S  G  N  I  D  T  A  W  S  N  L    74

 241 ACCCACCCGGGGAACAAGATCAACCTCCTCGGCTTCTTGGGCCTTGTCCACTGCCTTCCC   300
  75 T  H  P  G  N  K  I  N  L  L  G  F  L  G  L  V  H  C  L  P    94

 301 TGCAAAGATTCGTGCGACGGCGTGGAGTGCGGCCCGGGCAAGGCGTGCCGCATGCTGGGG   360
  95 C  K  D  S  C  D  G  V  E  C  G  P  G  K  A  C  R  M  L  G   114

 361 GGCCGCCCGCGCTGCGAGTGCGCGCCCGACTGCTCGGGCTCCCGGCGCGGTTGCAGGTC   420
 115 G  R  P  R  C  E  C  A  P  D  C  S  G  L  P  A  R  L  Q  V   134
                 *

 421 TGCGGCTCAGACGGCGCCACCTACCGCGACGAGTGCGAGCTGCGCGCCGCGCGCTGCCGC   480
 135 C  G  S  D  G  A  T  Y  R  D  E  C  E  L  R  A  A  R  C  R   154

 481 GGCCACCCGGACCTGAGCGTCATGTACCGGGGCCGCTGCCGCAAGTCCTGTGAGCACGTG   540
 155 G  H  P  D  L  S  V  M  Y  R  G  R  C  R  K  S  C  E  H  V   174

 541 GTGTGCCCGCGGCCACAGTCGTGCGTCGTGGACCAGACGGGCAGCGCCCACTGCGTGGTG   600
 175 V  C  P  R  P  Q  S  C  V  V  D  Q  T  G  S  A  H  C  V  V   194

 601 TGTCGAGCGGCGCCCTGCCCTGTGCCCTCCAGCCCCGGCCAGGAGCTTTGCGGCAACAAC   660
 195 C  R  A  A  P  C  P  V  P  S  S  P  G  Q  E  L  C  G  N  N   214
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FIG. 1A

Follistatin-3

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     #
661  AACGTCACCTACATCTCCTCGTGCCACATGCGCCAGGCCACCTGCTTCCTGGGCCGCTCC  720
215  N  V  T  Y  I  S  S  C  H  M  R  Q  A  T  C  F  L  G  R  S   234
                                    *
721  ATCGGCGTGCGCCACGCGGGCAGCTGCGCAGGCACCCCTGAGGAGCCGCCAGGTGGTGAG  780
235  I  G  V  R  H  A  G  S  C  A  G  T  P  E  E  P  P  G  G  E   254
     *
781  TCTGCAGAAGAGGAAGAGAACTTCGTGTGAGCCTGCAGGACAGGCCTGGGCCTGGTGCCC  840
255  S  A  E  E  E  E  N  F  V                                     263

841  GAGGCCCCCCATCATCCCCTGTTATTTATTGCCACAGCAGAGTCTAATTTATATGCCACG  900

901  GACACTCCTTAGAGCCCGGATTCGGACCACTTGGGGATCCCAGAACCTCCCTGACGATAT  960

961  CCTGGAAGGACTGAGGAAGGGAGGCCTGGGGGCCGGCTGGTGGGTGGGATAGACCTGCGT 1020

1021 TCCGGACACTGAGCGCCTGATTTAGGGCCCTTCTCTAGGATGCCCCAGCCCCTACCCTAA 1080

1081 GACCTATTGCCGCGGAGGATTCCACACTTCCGCTCCTTTGGGGATAAACCTATTAATTAT 1140

1141 TGCTACTATCAAGAGGGCTGGGCATTCTCTGCTGGTAATTCCTGAAGAGGCATGACTGCT 1200

1201 TTTCTCAGCCCCAAGCCTCTAGTCTGGGTGTGTACGGAGGGTCTAGCCTGGGTGTGTACG 1260

1261 GAGGGTCTAGCCTGGGTGAGTACGGAGGGTCTAGCCTGGGTGAGTACGGAGGATCTAGCC 1320

1321 TGGGTGAGTACGGAGAGTCTAGCCTGGGTGTGTATGGAGGATCTAGCCTGGGTGAGTATG 1380

1381 GAGGGTCTAGCCTGGGTGAGTATGGAGGGTCTAGCCTGGGTGTGTATGGAGGGTCTAGCC 1440

1441 TGGGTGAGTATGGAGGGTCTAGCCTGGGTGTGTATGGAGGGTCTAGCCTGGGTGAGTATG 1500

1501 GAGGGTCTAGCCTGGGTGTGTACGGAGGGTCTAGTCTGAGTGCGTGTGGGGACCTCAGAA 1560

1561 CACTGTGACCTTAGCCCAGCAAGCCAGGCCCTTCATGAAGGCCAAGAAGGCTGCCACCAT 1620
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FIG. 1B

Follistatin-3

1621 TCCCTGCCAGCCCAAGAACTCCAGCTTCCCCACTGCCTCTGTGTGCCCCTTTGCGTCCTG 1680

1681 TGAAGGCCATTGAGAAATGCCCAGTGTGCCCCCTGGGAAAGGGCACGGCCTGTGCTCCTG 1740

1741 ACACGGGCTGTGCTTGGCCACAGAACCACCCAGCGTCTCCCCTGCTGCTGTCCACGTCAG 1800

1801 TTCATGAGGCAACGTCGCGTGGTCTCAGACGTGGAGCAGCCAGCGGCAGCTCAGAGCAGG 1860

1861 GCACTGTGTCCGGCGGAGCCAAGTCCACTCTGGGGGAGCTCTGGCGGGGACCACGGCCA 1920

1921 CTGCTCACCCACTGGCCCCGAGGGGGGTGTAGACGCCAAGACTCACGCATGTGTGACATC 1980

1981 CGGAGTCCTGGAGCCGGGTGTCCCAGTGGCACCACTAGGTGCCTGCTGCCTCCACAGTGG 2040

2041 GGTTCACACCCAGGGCTCCTTGGTCCCCCACAACCTGCCCCGGCCAGGCCTGCAGACCCA 2100

2101 GACTCCAGCCAGACCTGCCTCACCCACCAATGCAGCCGGGGCTGGCGACACCAGCCAGGT 2160

2161 GCTGGTCTTGGGCCAGTTCTCCCACGACGGCTCACCCTCCCCTCCATCTGCGTTGATGCT 2220

2221 CAGAATCGCCTACCTGTGCCTGCGTGTAAACCACAGCCTCAGACCAGCTATGGGGAGAGG 2280

2281 ACAACACGGAGGATATCCAGCTTCCCCGGTCTGGGGTGAGGAGTGTGCGGGAGCTTGGGCA 2340

2341 TCCTCCTCCAGCCTCCTCCAGCCCCCAGGCAGTGCCTTACCTGTGGTGCCCAGAAAAGTG 2400

2401 CCCCTAGGTTGGTGGGTCTACAGGAGCCNCAGCCAGGCAGCCCACCCCACCCTGGGGCCC 2460

2461 TGCCTCACCAAGGAAATAAAGACTCAAAGAAGCCT 2495

FIG. 1C

Percent Similarity: 61.508 Percent Identity: 43.254

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x
Follistatin1.aa

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 51 SKEECCSTGRLSTSWTE..EDVNDNTLFKWMIFNGGAPNCIPCKETCENV 98

103 ECGPGKACRM.LGGRPRCECAPDCSGLPARLQVCGSDGATYRDECELRAA 151
 99 DCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNECALLKA 148

152 RCRGHPDLSVMYRGRCRKSCEHVVCPRPQSCVVDQTGSAHCVVCRAAPCP 201
149 RCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRI.CP 197

202 VPSSPGQELCGNNNVTYISSCHMRQATCPLGRSIGVRHAGSC..AGTPEE 249
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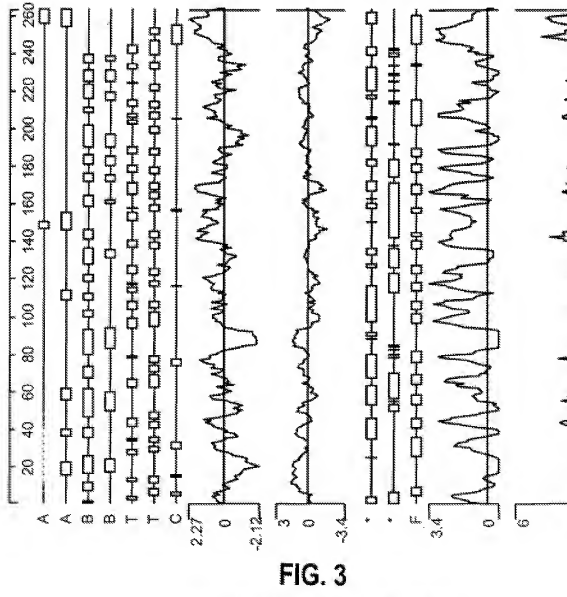
FIG. 2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Alpha, Regions - Chou-Fasman
Beta, Regions - Garnier-Robson
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Turn, Regions - Garnier-Robson
Turn, Regions - Chou-Fasman
Coil, Regions - Garnier-Robson
Hydrophilicity Plot - Kyte-Doolittle
Hydrophobicity Plot - Hopp-Woods
Alpha, Amphipathic Regions - Eisenberg
Beta, Amphipathic Regions - Eisenberg
Flexible Regions - Karplus-Schulz
Antigenic Index - Jameson-Wolf
Surface Probability Plot - Emini

FIG. 3

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Glu Cys Cys Ala Ser Gly Asn Ile Asp Thr Ala Trp Ser Asn Leu Thr
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cac ccg ggg aac aag atc aac ctc ctc ggc ttc ttg ggc ctt gtc cac   291
His Pro Gly Asn Lys Ile Asn Leu Leu Gly Phe Leu Gly Leu Val His
    50                  55                  60                  65

tgc ctt ccc tgc aaa gat tcg tgc gac ggc gtg gag tgc ggc ccg ggc   339
Cys Leu Pro Cys Lys Asp Ser Cys Asp Gly Val Glu Cys Gly Pro Gly
                70                  75                  80

aag gcg tgc cgc atg ctg ggg ggc cgc ccg cgc tgc gag tgc gcg ccc   387
Lys Ala Cys Arg Met Leu Gly Gly Arg Pro Arg Cys Glu Cys Ala Pro
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gac tgc tcg ggg ctc ccg gcg cgg ttg cag gtc tgc ggc tcg gac ggc   435
Asp Cys Ser Gly Leu Pro Ala Arg Leu Gln Val Cys Gly Ser Asp Gly
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gcc acc tac cgc gac gag tgc gag ctg cgc gcc gcg cgc tgc cgc ggc   483
Ala Thr Tyr Arg Asp Glu Cys Glu Leu Arg Ala Ala Arg Cys Arg Gly
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cac ccg gac ctg agc gtc atg tac cgg ggc cgc tgc cgc aag tcc tgt   531
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Gly Ser Ala His Cys Val Val Cys Arg Ala Ala Pro Cys Pro Val Pro
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tcc agc ccc ggc cag gag ctt tgc ggc aac aac aac gtc acc tac atc   675
Ser Ser Pro Gly Gln Glu Leu Cys Gly Asn Asn Asn Val Thr Tyr Ile
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tcc tcg tgc cac atg cgc cag gcc acc tgc ttc ctg ggc cgc tcc atc   723
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Ser Leu Val Leu Gln Thr Asp Val Thr Arg Ala Glu Cys Cys Ala Ser
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Gly Asn Ile Asp Thr Ala Trp Ser Asn Leu Thr His Pro Gly Asn Lys
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Ile Asn Leu Leu Gly Phe Leu Gly Leu Val His Cys Leu Pro Cys Lys
55                  60                  65                  70

Asp Ser Cys Asp Gly Val Glu Cys Gly Pro Gly Lys Ala Cys Arg Met
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Leu Gly Gly Arg Pro Arg Cys Glu Cys Ala Pro Asp Cys Ser Gly Leu
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Pro Ala Arg Leu Gln Val Cys Gly Ser Asp Gly Ala Thr Tyr Arg Asp
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Glu Cys Glu Leu Arg Ala Ala Arg Cys Arg Gly His Pro Asp Leu Ser
    120                 125                 130

Val Met Tyr Arg Gly Arg Cys Arg Lys Ser Cys Glu His Val Val Cys
135                 140                 145                 150

Pro Arg Pro Gln Ser Cys Val Val Asp Gln Thr Gly Ser Ala His Cys
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Val Val Cys Arg Ala Ala Pro Cys Pro Val Pro Ser Ser Pro Gly Gln
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Glu Leu Cys Gly Asn Asn Asn Val Thr Tyr Ile Ser Ser Cys His Met
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Arg Gln Ala Thr Cys Phe Leu Gly Arg Ser Ile Gly Val Arg His Ala
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gcctctttnt gcccctttgc ntcctgtgaa ggccattgag aaatgcccag tgtgcccrct 180

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tttcaaacca gctatgggga gaggacaaca cggaggatat tccagcttcc ccggtctggg 180

grgaaggagt gtggggagcn tgggncatcc tcctccagtn tcctccagcc cccaggnagt 240

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ccagcccaag actccagttc crcactgcct ctgtgtgccc tttgcgtcct gtgaagccat 180

tgagaaatgc ccatgtgccc ctgggaaagg gcacggctgt gtcctgacag ggtgtgtttg 240

cacagaccac caggtttcct gtgtgtcagt attatgagga acgtcggtgn ttagagtnga 300

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<210> 16
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<212> DNA
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<223> Description of Artificial Sequence: hemaglutanin
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<223> Description of Artificial Sequence: Myc tag

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<210> 22
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<212> PRT
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<220>
<223> Description of Artificial Sequence: Myc tag

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1 5 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/19198

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12P 21/06, 21/02, 21/04; A61K 38/00, 38/16
US CL : 435/69.1, 69.4, 252.3; 536/23.1, 23.5, 935/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/69.1, 69.4, 252.3, 536/23.1, 23.5, 935/22

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Genetic Databases: Issued Patents; SPTREMBL12; EST; GenEmbl

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P -- Y,P	US 5,942,420 A (HOLTZMAN) 24 August 1999, entire document, especially Col. 55 & 56, SEQ ID NO: 2.	1-6 --- 6-21
X	Database GenEmbl on STN, AN AA375541. ADAMS,M.D. "Initial assessment of human gene diversity and expression patterns based upon 83 million nucleotides of cDNA sequence". Nature. 1995. Vol. 377 (6547 Suppl), pages 3-174 (See EST787916).	21
X	HAYETTE et al. FLRG (follistatin-related gene), a new target of chromosomal rearrangement in malignant blood disorders. Oncogene. 1998, Vol. 16, No. 22, pages 2949-2954, see entire document and attached database.	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 September 2000 (14.09.2000)	14 NOV 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Patricia Robinson Telephone No. 703-308-0196

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